Taiwanese Journal of Obstetrics & Gynecology 59 (2020) 882–890

Contents lists available at ScienceDirect

Taiwanese Journal of Obstetrics & Gynecology

journal homepage: www.tjog-online.com

Original Article

Retinoic acid and fibroblast growth factor-2 play a key role on modulation of sex hormones and apoptosis in a mouse model of polycystic ovary syndrome induced by estradiol valerate

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ARTICLE INFO

Article history:
Accepted 1 July 2020

Keywords:
Retinoic acid
Fibroblast growth factor-2
PCOS
Apoptosis
Mouse

ABSTRACT

Objective: The main goal of the present study is to investigate the effects of retinoic acid and fibroblast growth factor-2 on serum levels of FSH and LH, histology, and apoptosis in the mouse model of Poly Cystic Ovary Syndrome (PCOS).

Materials and methods: 80 female NMRI mice have been randomly divided into eight groups. Group 1 received normal saline as a control, and Group 2 received estradiol valerate (EV) at 4 mg/100 g of body weight. Moreover, Groups 3–4 were administered with RA (a dose of 0.05 μg/μl) and FGF2 (a dose of 0.01 μg/kg), respectively. Groups 5 and 6 respectively received the EV plus the RA (0.05 μg/μl) and FGF2 (0.01 μg/kg). Group 7 received the RA and FGF2 at doses corresponding to healthy mice, and Group 8 received the EV plus the RA + FGF2 (similar to previous doses). RA and FGF2 were injected three times per week for four weeks. Finally, histological and immunohistological parameters of the ovary were evaluated.

Results: The study revealed that both single and combined injection of fibroblast growth factor-2 (FGF2) and retinoic acid (RA) in groups 5, 6, and 8 significantly reduced follicular diameters compared to group 2. Measurements confirmed that simultaneous injection of RA and FGF2 into polycystic mice significantly increased antral follicles, corpus luteum (CL), epithelial thickness, and oocyte diameter as well as decreased cystic follicles. Positive TUNEL cells that were considerably increased in the antral follicle of group 2 significantly decreased in the RA and FGF2 recipient groups, either alone or in combination. Besides, the injection of FGF2 increased preantral follicles and CL.

Conclusion: The findings of the present investigation reveal that injection of RA and FGF2 has both protective and ameliorative effects that can promise new therapies for women with PCOS.

Introduction

Poly Cystic Ovarian Syndrome (PCOS) is known to be the main reason for infertility in women (20% of couples with infertility) [1]. Andrology symptoms of PCOS include: menstrual disorders (Oligomenorrhea and Amenorrhea), follicle-stimulating hormone (FSH) and decreased or increased secretion of luteinizing hormone (LH), histological abnormalities include bilateral enlargement of the ovary (approximately more than 10 mm), increase in ovarian follicles with small diameter (approximately 10 mm) around the stroma, enlarged follicle overhang and ovarian stroma and consequently reduced (or no) ovulation and infertility [2]. In PCOS patients, ovulation failure occurs at the time of ovulation. In ovulation of the PCOS patients, matrix metallopeptidases (MMPs) that break

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https://doi.org/10.1016/j.tjog.2020.08.004

down collagen increase and Lysyl Oxidase (LOX) decreases as well as in response to androgen uptake or an increase in testosterone production. MMPs are types of proteolytic enzymes that have significant responsibility for remodeling the extracellular matrix during ovarian follicular growth as well as ovulation. RA regulated morphogenesis of ovarian tissue through *MMPs* expression [3]. MMP2 activity is significantly reduced, and LOX activity is increased, thereby inhibiting collagen digestion and follicle rupture, leading to non-ovulation in these patients [4].

Retinoids are a group of small organic molecules that play a critical role in some developmental and biological processes, including morphogenesis, growth, and cell differentiation [5]. Vitamin A deficiency can lead to a wide range of reproductive disorders in various species, such as ovarian and testicular size decline [6]. Studies on rats have shown that vitamin A deficiency in the diet during pregnancy leads to placental apoptosis [7]. Some studies have shown that retinol prevents fetal atrophy if retinol is injected into the rat model with vitamin A deficiency before 10 days of pregnancy [8].

Fibroblast growth factor-2 (FGF-2) is a factor involved in paracrine signaling inside the follicle. The fibroblast growth factor is a group of specific heparin-binding polypeptides that takes a leading role in the development, cell growth, tissue repair, and transport. In some in-vitro investigations, it has been indicated that FGF-2 improved proliferation of theca cells and granulosa and the growth of primordial and primary follicles. The differentiation of ovarian granulosa cells was performed by fibroblast growth factor [9] through the expression of luteinizing hormone (LH) receptors within granulosa cells and proliferating ovarian germ cells [10]. Fibroblast growth factor-2 (FGF2) contributes to the regulation of a significant number of ovarian functions such as granulosa cell mitosis [11] and granulosa cell differentiation and apoptosis [12]. Many studies show that adding 5 ng/ml of FGF2 to the embryonic stem cell culture medium increases the expression level of genes involved in pluripotency in cells [13]. Zhang et al. (2012) added 0.5, 5 and 50 ng/ml (ng/ml) FGF2 to primary bovine follicles and their results revealed that adding different doses of FGF2 to the IVM medium of primary bovine follicles increased the secondary oocyte, decreased apoptosis of follicular granulosa cells and increased blastocyst formation at day seven after IVF [14]. An investigation by Tahaei et al. (2011) indicated that adding 2 μl of retinoic acid to the culture medium of primary follicles increased the maturation rate of these follicles and the percentage of oocyte maturation, and also increased the number of blastocysts fertilized [15]. Pu et al. (2014) indicated that 10 and 100 nmol/L of all-trans retinoic acid (ATRA) improved oocyte survival and nuclear maturation compared to controls. The researchers reported that ATRA inhibits apoptosis in cumulus cells by increasing the expression of Bcl-2 and catalase (CAT) genes and decreasing caspase-8 expression [16]. Hence, the assumption is that administrating RA and FGF2 is probably an efficient and excellent alternative in the survival and follicular development in ovarian tissue and improving PCOS induction by estradiol valerate. For this purpose, both immunohistochemistry and histological investigations of apoptosis in granulosa cells of ovarian follicles have been conducted to study and compare the morphology of follicles pre- and post-treatment for four weeks in the existence or non-existence of RA, FGF2 or the association of RA and FGF2.

Materials and methods

Animals

Eighty immature female NMRI mice aged four weeks (Pasteur Institute, Tehran, Iran) weighing 20 g were used. The mice were preserved under 25 °C ambient temperature, 50% humidity, and a daily rhythmic light–dark cycle.

All mice were allowed ad libitum and water access. Ethics Committee of Shahrekord University of Medical Sciences (Ethic number IR. SKUMS. REC. 1397. 098) approved all procedures in the present study.

Experimental design

Female mice have been divided into eight groups randomly (10 mice in each group):

Group1 (Normal Control): Received normal saline only.

Group2 (Positive Control): Only estradiol valerate is given.

Group3 (RA Control Group): Only injection of RA at a dose of 0.05 μg/μl.

Group4 (FGF2 Control Group): Only injection of FGF2 at a dose of 0.01 μg/kg.

Group5 (RA Intervention Group): RA injection at a dose of 0.05 μg/μl to mice induced PCOS.

Group6 (FGF2 Intervention Group): Injection of FGF2 at a dose of 0.01 μg/kg to mice induced PCOS.

Group7 (RA + FGF2 Control group): Simultaneous injection of RA and FGF2 at doses corresponding to healthy mice.

Group8 (RA + FGF2 Intervention group): Simultaneous injection of RA and FGF2 at doses corresponding to PCOS-induced mice.

Ovarian phase synchrony

Male vasectomized mice were housed in female cages for three days to cross-cycle the mice. In order to obtain a smear, a cotton swab dipped in saline with circular motions was drawn on the vaginal wall. The sample was then put on the slide and fixed with 96% alcohol for 20 min. The slides were exposed to air to dry and finally stained using the Papanicolaou method.

Induction method of PCOS

In order to induce the phenotype of PCOS, animals were treated subcutaneously for 20 days with a daily injection of 4 mg/kg estradiol valerate (Aburaihan Pharmaceutical Co., Tehran, Iran), which was dissolved in sesame oil and normal saline (0.9%) [17]. In order to confirm the induction of PCOS, after 60 days, three mice were sacrificed, and the ovaries were removed from the oviduct and placed in a container containing PBS solution. Excess fats were carefully removed without damage to the ovarian tissue under the loop, and the ovaries were inserted into the paraformaldehyde fixative for histopathology [18].

Administration of RA and FGF2

RA was dissolved in dimethyl sulfoxide (DMSO) then the amount of 0.05 μg/μl body weight was injected intraperitoneally three times weekly for four weeks [19]. FGF2 was dissolved in saline (0.9%) [20] and injected intraperitoneally at a dose of 0.01 μg/μl three times each week for four weeks [21].

Measuring sex hormones

In order to assess FSH and LH levels on the day following the last injection and overnight fasting, animals were anesthetized with ether, then 2 ml of blood was collected from the heart with an insulin syringe and transported to clot activator separation gel tube. Then samples have been centrifuged at 3000 rpm for five minutes, and serum was separated. Thereafter, the level of LH and FSH measured mouse LH using standard enzyme-linked

immunosorbent assay (ELISA) Kit (Antibodies-online; Aachen, Germany, catalog no.: ABIN415551) and mouse FSH ELISA Kit (antibodies-online; Aachen, Germany, catalog no.: ABIN1873388) respectively. 50 μL of serum was added to each well. 50 μL of detection reagent A added to wells immediately and placed on a plate shaker. Furthermore, the wells were incubated for 1 h at 37 °C and aspirate all wells, as well as plate, washed three times. 100 μL of detection reagent B added and incubated for 30 min at 37 °C and five times washed. After adding a 90 μL substrate solution, the wells were incubated 20 min at 37 °C. Thereafter adding 50 μL of stop solution to wells was readied by ELISA reader at 450 nm immediately [22].

Histological examination

The mice ovaries were removed aseptically as well as washed with sterile PBS for histological examination. After samples were soaked in 4% paraformaldehyde (Sigma, St. Louis, MO, USA) overnight, then were dehydrated in different ascending concentrations of ethanol (at 2% concentration it was done twice, but at 100% concentration two consecutive times were performed) manually. In the following, tissues clarified with xylene, embedded in paraffin, and cut into 5 μm-thick sections with a rotary microtome (Leica, Wetzlar, Germany). Tissue areas have been stained with common hematoxylin-eosin (Sigma–Aldrich; Merck KGaA) and were cleared with xylene. Afterward, sections were observed under light microscopy (Olympus, Tokyo, Japan) with 100× magnification, and the number of follicles counted in different groups. In order to evaluate ovarian tissue changes, the structures in the ovary were morphologically divided into six groups, according to the Sun (2015) method [23]. The follicular stages were classified as follows: 1) Primordial follicles, 2) Primary follicles, 3) Secondary or preantral follicles, 4) Graffian or antral follicles, 5) Cystic follicles, and 6) Corpus luteum (CL).

Morphometrical assessment

The specimens were examined under a microscope, and follicular diameter (μm), oocyte diameter (μm), theca thickness (μm), ovarian volume (mm^3), and epithelium thickness (μm) were measured with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) [24].

Evaluation of apoptosis by TUNEL assay

For proving apoptosis in individual cells, the apoptosis kit (Cell Death Detection Kit; POD, Roche, Germany) was used applying the TdT mediated dUTP nick end labeling (TUNEL) assay. The approach is a key indicator extensively used for the assessment of apoptosis-induced DNA fragmentation. After the paraffin-embedded block of ovarian tissue in all groups, 5-μm serially sections were prepared. These slices were mounted on poly-L-lysine-coated glass slides and also deparaffinized and rehydrated using descending ethanol concentrations and distilled water. In order to confirm the accuracy of the positive control samples, the microwave irradiation (MW) pretreatment method was used before enzymatic labeling. In this method, tissue sections are placed in a 700 W oven for 10 min. In order to block the endogenous peroxidase before enzymatic labeling, slices were incubated in PBS containing 3% hydrogen peroxide (H_2O_2) for 10 min. Then, the sections have been incubated with TUNEL reaction solution containing terminal deoxynucleotidyl transferase (TdT) as well as fluorescein-dUTP for 60 min at 37 °C. Then, the sections have been washed three times in PBS solution and incubated with anti-fluorescence antibody conjugated with peroxidase (Roche Applied Science, Mannheim, Germany) at 37 °C for 30 min. Afterward, the sections have been washed again in PBS solution three times and stained with chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma–Aldrich, Germany) [25]. The average number of apoptotic cells that turned brown was calculated by ImageJ software [26]. In all groups, antral follicles and primary oocytes inside them were used to measure the diameter and thickness as well as to evaluate cell apoptosis.

Data analysis

The authors imported the data into SPSS version 20. The variables in the study groups were compared using one-way ANOVA with Tukey post hoc test, and P-value less than 0.05 was considered as significant.

Results

Verification of the PCOS mice model

The successful induction of syndrome after 20 days of induction has been investigated using light microscopy for assessing the animals' estrus cyclicity with regard to the vaginal epithelial cell smears. Moreover, a number of the animals from the normal and PCOS groups have been killed at every period of estrous and proestrus cycle, and the ovaries have been harvested for histopathology evaluation for the purpose of providing a detailed model verification. All animals were in the metestrus phase of the sexual cycle. Vaginal smears showed that the mice were in the metestrus phase (Fig. 1).

Results of the number of ovarian follicles and ovarian volume

Different control follicles were seen in the ovarian tissue of mice. Ovaries of the other groups showed variable numbers of follicles at different developmental stages as well as cystic follicles with an extremely thin layer of granulosa cells (Fig. 2). No statistically significant difference was observed among the study groups ($P > 0.05$) with regard to PMF. The results showed that estradiol valerate injection for 20 consecutive days (group 2) compared with the control group (group 1), led to the decrease of PF ($P = 0.004$), an increase of preantral follicles ($P < 0.05$), an increase of cystic follicle ($P < 0.05$), and the reduction in the number of CL ($P < 0.05$). Results reveal that the injection of 0.05 μg/μl retinoic acid dose to healthy mice (group 3) compared to the control group (group 1) led to the increase of antral ($P < 0.05$) and preantral ($P = 0.001$) follicles. The injection of FGF2 at a dose of 0.01 μg/kg to healthy mice (group 4) compared to the control group (group 1) led to an increase of preantral follicles ($P = 0.018$) and CL ($P = 0.019$). The injection of 0.05 μg/μl retinoic acid into polycystic mice (group 5) significantly increased the preantral follicles and reduced the cystic follicles ($P < 0.05$). In group 6, numerous antral follicles and the CL were increased in comparison with group 2 ($P < 0.05$and $P = 0.001$, respectively), whereas cystic follicles decreased ($P < 0.05$). In comparison with group 1 in group 7, the number of preantral, antral, and CL follicles were increased ($P < 0.05$), indicating a high efficacy of concomitant injection of RA and FGF2 into healthy mice to increase ovarian follicle maturation. Results revealed that simultaneous injection of RA and FGF2 into polycystic mice (group 8) compared to group 2 led to an increase of antral follicles and CL and a decrease of cystic follicles ($P < 0.05$) (Fig. 2). According to Fig. 3, the ovarian volume has been indicated to be compared between the RA and FGF2 groups with the PCOS group. The total volume of ovarian tissue in the treatment groups was remarkably increased in comparison to the PCOS group ($p < 0.01$) (Fig. 3). Also,

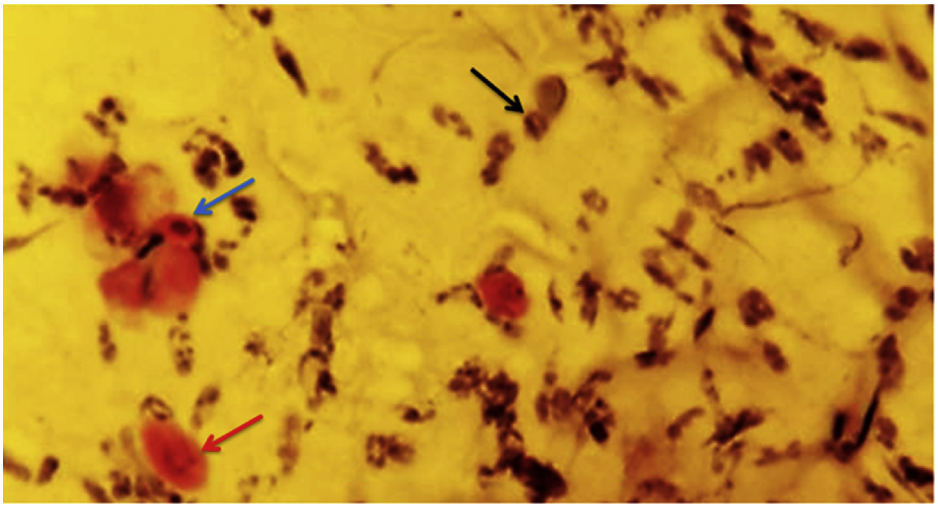

Fig. 1. The mice metestrus phase shows nucleated epithelial cells (blue arrow), non-nucleated epithelial cells (red arrow), and leukocytes (black arrow).

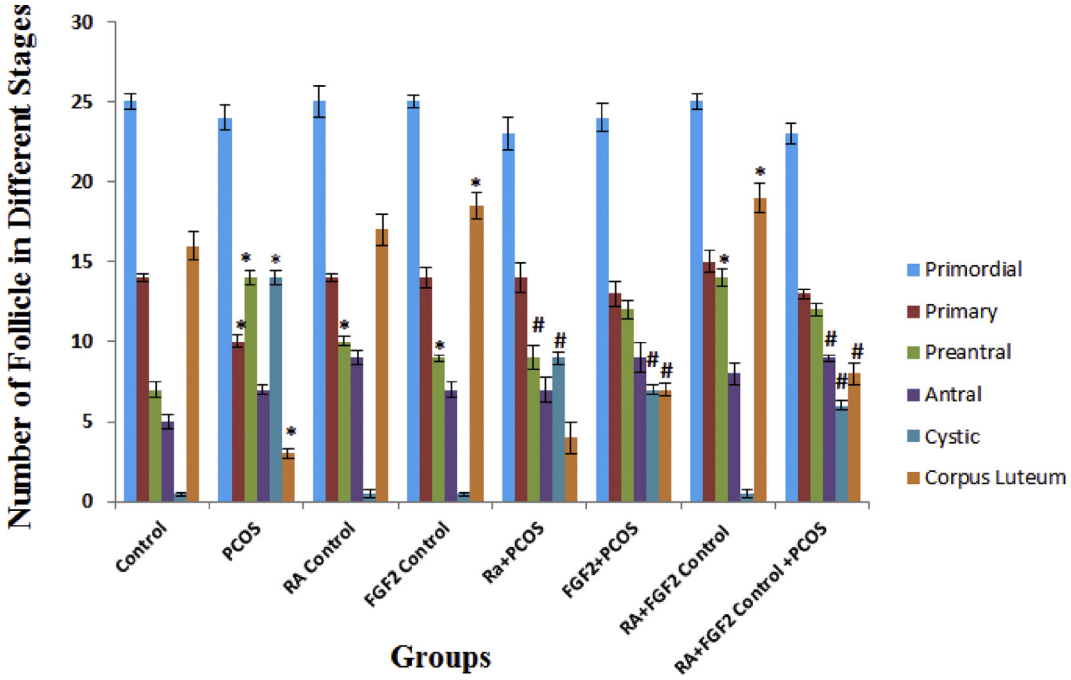

Fig. 2. Comparison of follicles at different developmental stages in studied groups. In this graph, the asterisk (*) demonstrates a significant difference with group 1 ($P < 0.05$) and **#** sign shows significant difference with groups 2 ($P < 0.05$).

there was a difference between 5 and 6 groups compared to the control groups. Nonetheless, these results were not significant (Fig. 3).

Quantitative analysis of follicle and oocyte (diameter and thickness)

The follicular diameter was increased ($P < 0.05$) in PCOS mice (group 2) compared to group 1. Results show that either single or combined injection of RA and FGF2 in groups 5, 6, and 8 significantly reduced the follicular diameter compared to group 2 ($P = 0.042$, $P = 0.026$, and $P = 0.008$, respectively) (Fig. 4). The measurements performed with ImageJ software showed that the thickness of the epithelium (granulosa cell thickness) was reduced in group 2 (PCOS-induced) ($P < 0.05$). Although this thickness in group 8 (simultaneous injection of RA and FGF2 into polycystic mice) was significantly increased ($P = 0.026$), in groups 5 and 6, single injection had no reversible and therapeutic effects ($p > 0.05$) (Fig. 4) (Table 1).

The diameter of the oocyte was reduced ($P < 0.05$) in PCOS mice (group 2) compared to group 1. The results showed that oocyte diameters in groups 4 and 7 were significantly higher than group 1 ($P < 0.05$). Besides, the results reveal that oocyte diameters were significantly increased in groups 6 and 8 than in group 2 ($P = 0.012$ and $P = 0.009$, respectively), which indicates reversible and therapeutic roles of FGF2 and RA-FGF2, respectively. Theca thickness in group 2 (PCOS mice) was abnormally increased in comparison to

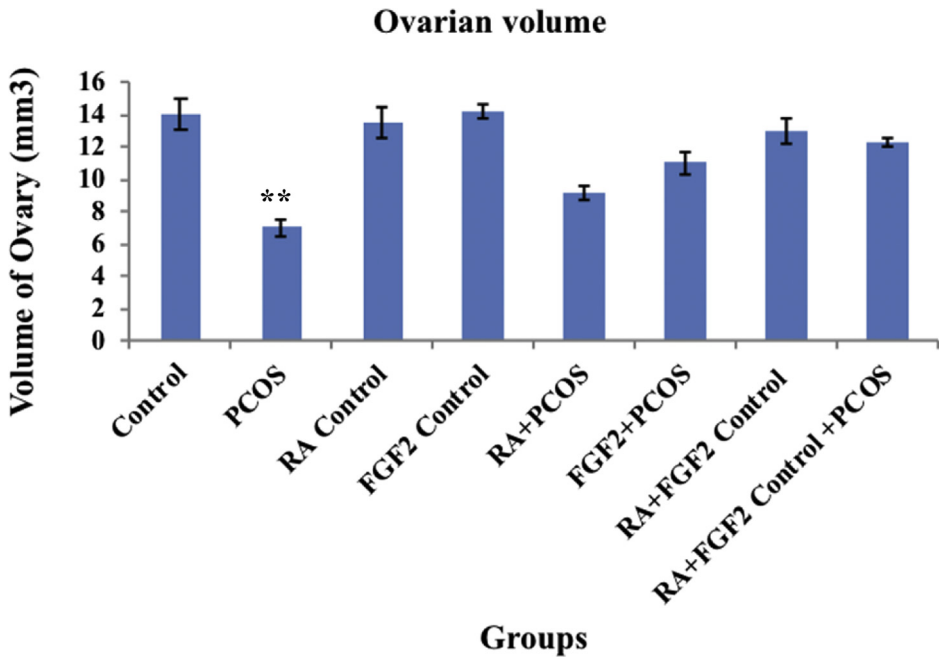

Fig. 3. Volume study. The effects of administration of RA and FGF2 on the volume of ovary (mm^3) of mice with PCOS. **P < 0.01 compared to treatment and control groups.

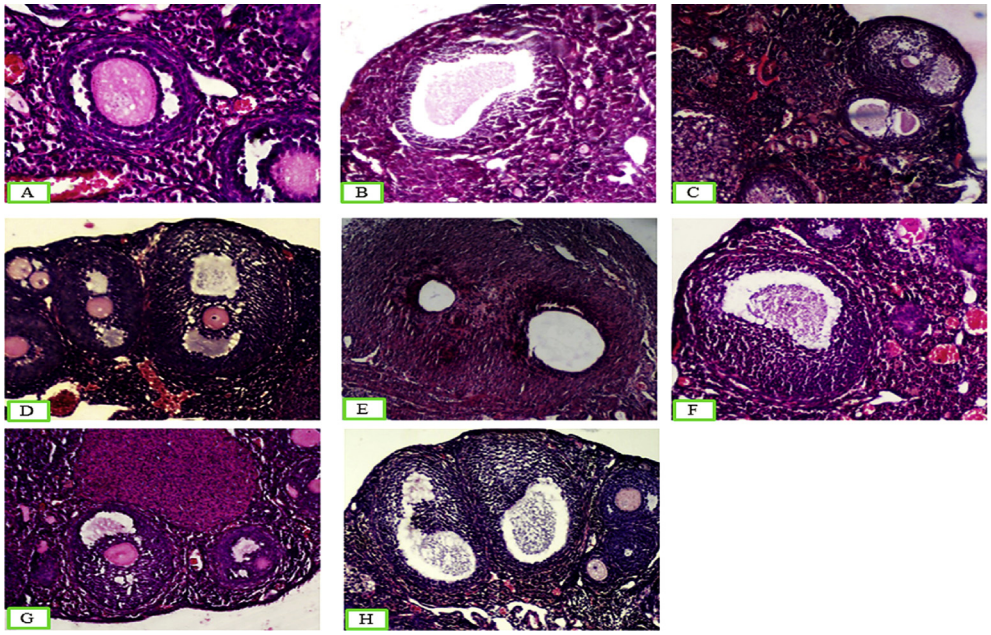

Fig. 4. Photomicrograph shows antral follicles in different studied groups. Images A (group 1), C (group 3), D (group 4), and G (group 7) represent that follicles are enclosed by a thick layer of granulosa cells and a normal theca layer. Image B (group 2) indicates that the follicle is enclosed by a thin layer of granulosa cells and a thicker theca layer compared to the control group and also an increase in the distance between granulosa cell layer and oocyte is observed. Images E (group 5), F (group 6), and H (group 8) show that the theca layer around the follicle is less thick than the PCOS group and provides better conditions for oocyte release which illustrated the features of a normal follicle image H, Hematoxylin and eosin (H&E) staining (100× magnification).

group 1 (P < 0.05), whereas this parameter significantly decreased in groups 6 and 8 compared to group 2 (P = 0.35 and P = 0.022, respectively) (Table 1).

Counting of apoptotic cells

Positive TUNEL cell counts indicate that the cell apoptosis in the antral follicle was determined by ImageJ software. In group 2, the quantity of positive TUNEL cells has been significantly raised compared to group 1 (P < 0.05). The number of apoptotic cells decreased in groups 5, 6, and 8 compared to group 2 (P = 0.035, P = 0.014, and P = 0.005, respectively) (Fig. 5) (Table 1).

Sex hormone measurement

The present study showed that FSH serum in group 2 was lower compared to the control group, which was significant (P < 0.05). Results showed that the injection of RA into healthy mice (group 3)

Table 1
Comparison of morphometrical parameters of follicle (or oocyte) and number of apoptotic granulosa cells in studied groups (Mean ± SD).

Groups	Granulosa epithelium thickness (μm)	Oocyte transverse diameter (μm)	Follicle transverse diameter (μm)	Theca layer thickness (μm)	Apoptotic cells (number)
1	11.48 ± 0.81	70.71 ± 3.1	168.7 ± 5.84	6.44 ± 1.08	2.2 ± 1.4
2	7.1 ± 1.19[a]	32.6 ± 2.91[a]	278.9 ± 6.1[a]	21.4±2[a]	50 ± 4.1[a]
3	11.6 ± 1.5	72.6 ± 3.06	164 ± 5.4	6.4 ± 0.96	1.9 ± 1.1
4	11.9 ± 1.1	58 ± 2.82[b]	170 ± 5.6	6.2 ± 1.54	1.4 ± 0.96
5	7.5 ± 1.08	34 ± 3.22	252±5[d]	19.4 ± 2.5	43.2 ± 4.2[d]
6	8.2 ± 1.31	41.8 ± 4.46[e]	246±6[e]	14.5 ± 1.7[e]	35.1 ± 3.17[e]
7	12.4 ± 1.57	82.2 ± 2.82[c]	165 ± 5.8	5.8 ± 1.03	1.4 ± 063
8	9 ± 1.33	45.6 ± 3.8[f]	234 ± 5.1[f]	13.3±2[f]	30.4 ± 2.2[f]

a, b, and c were significantly different to group 1 ($P < 0.05$); d, e, f were significantly different to group 2 ($P < 0.05$).

significantly decreased the FSH level than the control group ($P < 0.05$), but no significant difference was seen in serum FSH level in the RA injection group to PCOS mice (group 5) compared to group 2. In contrast, FGF2 injections into PCOS mice (group 6) led to an increase of FSH levels ($P = 0.016$), which indicates an improvement in hormone levels. ANOVA analysis showed a significant increase in serum LH in group 2 in comparison with group 1 ($P < 0.05$), and in groups 6 and 8, a significant difference has been observed in comparison with group 2 ($P < 0.05$). However, no significant statistical difference was seen among the other groups. ($P > 0.05$) (Fig. 6).

Discussion

According to microscopic observations of ovarian tissue, injections of estradiol valerate to mice lead to cystic follicles, decrease in antral follicles and CL, increase in LH and decrease FSH, increase in follicular diameter, decrease in epithelial thickness (decrease in granulosa cell thickness) and ovarian volume, decrease in oocyte diameter, and increase in theca thickness. The studies also revealed that injection of 0.05 μg/μl retinoic acid into healthy mice led to an increase of preantral and antral follicles and oocyte diameter. The results showed that both single and combined injection of RA and FGF2 in groups 5, 6, and 8 significantly reduced follicular diameters compared to group 2. The measurements showed that the simultaneous injection of RA and FGF2 into polycystic mice significantly led to an increase of antral follicles, CL, epithelial thickness, and oocyte diameter, and also the reduction of cystic follicles. Positive TUNEL cells that were significantly increased in the antral follicle of group 2 considerably decreased in the RA and FGF2 recipient groups, either alone or in combination. Besides, the injection of FGF2 to healthy mice at a dose of 0.01 μg/kg led to an increase of preantral follicles and CL. FGF2 also reduced the follicular theca thickness in PCOS mice. Since PCOS is an inflammatory process, factors such as tumor necrosis factor-alpha (TNF-α) are involved in studies showing that the increase in the theca layer of the follicle

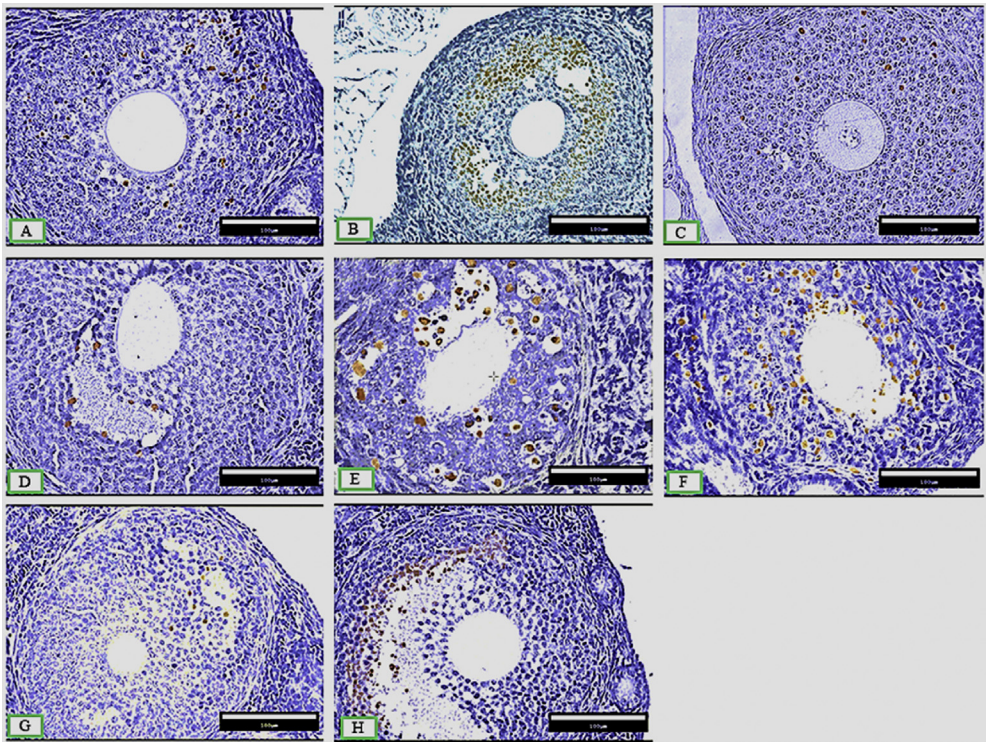

Fig. 5. Photomicrograph shows apoptosis of granulosa cells in the ovarian antral follicles. In order to determine the level of apoptosis of granulosa cells, the average signal intensity of TUNEL staining was measured automatically by ImageJ software. In group 2 (image B), the number of positive TUNEL cells was significantly increased compared to group 1 (image A). The decrease in apoptotic cells is seen in groups 5 (image E), 2 (image F), and 3 (image H). According to the data shown in image H, most peripheral granulosa cells are apoptotic and the corona radiata cells are healthy.

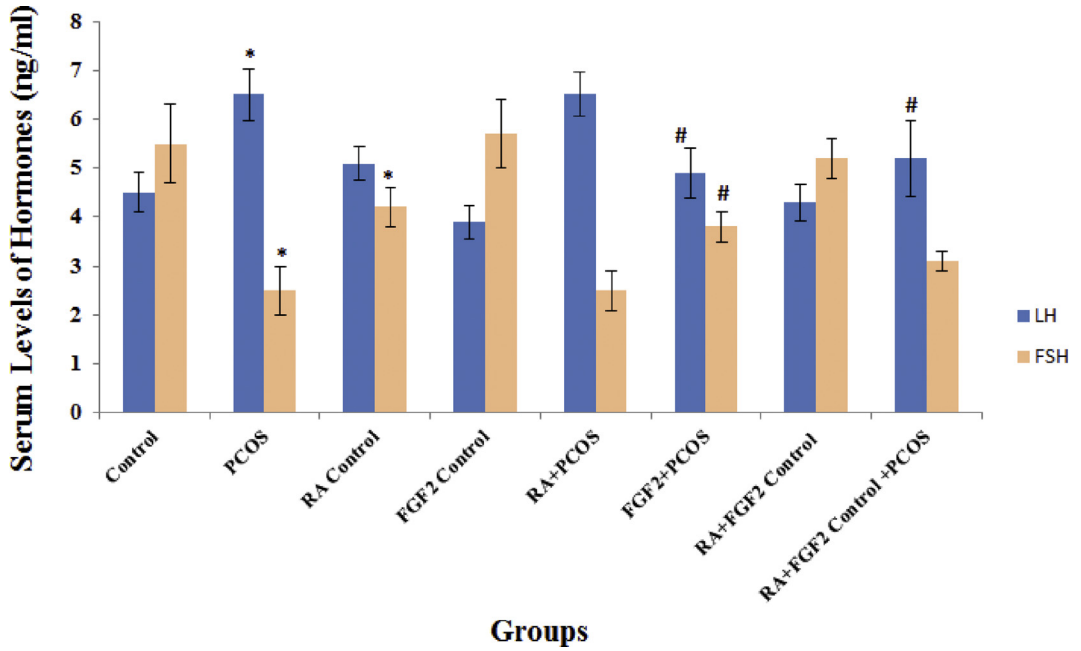

Fig. 6. The graph represents the levels of sex hormones including follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the studied groups. *: significantly differs with groups 1 ($P < 0.05$); #: significantly differs with groups 2 ($P < 0.05$).

and the significant decrease in the thickness of the granulosa layer occurs as a result of increased TNF- expression. TNF-α can induce Cyclooxygenase 2 (COX-2) gene transcription in different ways. TNF-α in human proliferate luteinizing granulosa cells and rats induces apoptosis of granulosa cells in the final antral follicles [27]. Fibroblast growth factors can alter the growth, development, and differentiation of reproductive tissues in different ways. In the female reproductive system, the effects of this factor are concentrated on the ovary in the present study, which indicates that the FGF2 factor stimulates granular cell production and reduces differentiation (reduced steroidogenesis) [28]. Understanding the relationship between signals that trigger and control the growth of ovarian follicles will be an essential improvement for the development of comprehensive in-vitro and in-vivo maturation techniques; FGF2 is one of which. Initial investigations showed the location of FGF2 in ovarian follicles and FGF2 receptors in growing follicles [29]. Studies reveal that the concentration of FGF2 ngmL50 allows the preservation of follicles active in the culture medium for five days. Given the interaction of RA and FGF2, it is suggested that RA activates functional receptors for FGF2 in mouse granulosa cells. FGF2 is effective in enhancing follicle diameter and granulosa cell proliferation, which was also observed in the present study. Furthermore, FGF2 stimulates the production of theca cells and cultured bovine stromal cells [30]. Each ovarian follicle exhibits specific cellular characteristics associated with its developmental stage. When the primary follicle contains one layer of granulosa cells, it still has no receptor for FSH, but since then, the receptors have appeared on the surface of the follicular cells. When a developing follicle passes through this stage, its survival and development depend on extracellular factors (pituitary hormones) [31], which was similar to the primary follicles in the groups in the present study. On the other hand, the mechanism of atresia inhibition and cystic follicle formation may be initiated by various factors such as pituitary surgery, diet disorder, environmental stressors, and chemicals [32]. Retinoic acid may influence oocyte maturation by affecting LH or FSH receptor expression of granulosa cells or may improve mRNA quality and promote oocyte maturation through increased polyadenylation [33]. Pauli et al. (2013) also reported that increased follicle size correlated with increased levels of retinol (ROL) and all-trans-retinoic acid (ATRA) levels in follicular fluid, and ATRA levels in follicles were considerably higher in healthy women in comparison with women with endometriosis. From these researchers' views, the presence of ATRA contributes significantly to the development and quality of oocytes and embryos, and the reduction of that results in IVF failure and infertility [34]. Retinol and its biological derivative, Retinoic Acid (RA), are necessary for numerous reproductive processes. ATRA acts by increasing B cell Lymphoma2 (BCL2) and catalase expression and reducing the caspase-8 expression and inhibits the apoptosis. The 9-CisRA and ATRA are physiologically active metabolites and natural components of vitamin A. Many investigations indicated that oocyte quality is enhanced by retinoic acid through several mechanisms such as oocyte protection against oxidative stress, FSH/LH receptor expression, and TNF-α reduction in oocyte alpha [35,36]. Various growth factors and inducers contribute to the development of ovarian follicles, and researchers are constantly exploring new inducers in this field. Jiang et al. indicated that adding fibroblast growth factors to the culture medium of ovarian granulosa cells decreased the expression level of mRNA encoding SPRY-3 and proapoptotic factor Bax. The Western blotting results of their study showed that FGF activates the ERK1/2 and Akt signaling pathway rapidly and transiently, thereby increasing the survival rate of granulosa cells, which play key roles in normal follicle maturation and egg quality [37]. FGF2 has the highest affinity for receptors. It causes cell proliferation and migration, protease secretion, and angiogenesis. Furthermore, FGF2 is capable of activating ribosomal DNA (rDNA) transcription, and its nuclear accumulation was linked to cell proliferation. Granulosa cells in the preantral and antral follicles can produce FGF2 at all stages of follicular development [38]. Some studies indicated that granulosa cell apoptosis is inhibited by FGF2. FGF2 induces granulosa cell survival by an unknown mechanism. However, there is evidence that FGF2 inhibits apoptosis through both genomic and acute functions. It is suggested that female ovarian volume has been partially and significantly affected by RA and FGF2 because of modulating reproductive endocrine functions and augmenting follicular development or

retaining primordial follicles. Acute functions appear to include normal levels of intracellular free calcium (Ca2þ). In granulosa cells, FGF2 controls Ca2þ levels through a protein kinase-dependent mechanism (PKCd). FGF2 involves the plasma membrane calcium ATPase enzyme (PMCA) to stimulate calcium release. The level of apoptosis in transgenic mouse granulosa cells was reduced by overexpression of FGF2. These studies indicate that FGF2 is a physiological regulator for granulosa cell survival [39,40]. However, little information is available on their role in oocyte maturation, ovarian folliculogenesis, and early stages of embryonic development [41]. Studies show that adding ATRA to theca cells enhances DHEA production. Thus, the retinoid family participates in the biosynthesis of androgens and the expression of steroidogenic enzymes in normal and PCOS cells [42].

Conclusion

Retinol plays an important role in ovarian steroidogenesis and female reproduction so that changing retinol to retinaldehyde increases secretion in PCOS cells compared to normal cells. That suggests that the enzymes responsible for retinol metabolism are found in single cells and are altered in PCOS individuals. Both RA and FGF2 could decrease DHEA and testosterone productions in PCOS thecal cells; therefore, the amount of retinol within the circulatory system in the PCOS model causes retinoid degradation in the ovaries. In this regard, the alleviation of tissue symptoms by both FGF2 and RA is probably due to the anti-inflammatory properties of this product. Finally, injections of RA and FGF2 into PCOS-induced mice have protective and potential modulator effects, which could promise new therapies for women with PCOS. In order to understand the signaling pathway and mediator molecules involving in RA and FGF2 effects in the PCOS model, it is recommended to perform molecular techniques and electron microscopic evaluation.

Ethical approval and consent to participate

The authors declare that the research Ethics Committee at Shahrekord University of Medical Sciences, Shahrekord, Iran (IR. SKUMS. REC. 1397. 098) approved all the protocols mentioned earlier in the present study. All methods were regulated and performed following relevant guidelines and research instructions.

Consent for publication

All authors have contributed to the writing of this article.

Availability of supporting data

The authors stated supporting data would be made available to others on request.

Funding sources

School of Medicine, Shahrekord University of Medical Sciences, Shahrekord, Iran financially supported the study.

Authors' contributions

A.M designed this study, and A.R conducted the histological study and drafted the manuscript. MJ.R and AR provided the clinical data and sample and helped to draft the manuscript. R.A, A.R, and A.M. carried out the animal model and performed the statistical analysis. A. R conducted the molecular test, and A.M carried out the immunohistochemistry. All authors read and approved the final manuscript.

Declaration of Competing Interest

There is no conflict of interest, and all authors have read and approved the manuscript being submitted.

Acknowledgment

The authors appreciate the financial support for this study by the Research Council of Shahrekord University of Medical Sciences with grant number of “1396-08-75-3662”.

Abbreviations

PCOS	Polycystic Ovary Syndrome
EV	Estradiol Valerate
RA	Retinoic Acid
FGF2	Fibroblast Growth Factor-2
CL	Corpus Luteum
LH	Luteinizing Hormone
FSH	Follicle-stimulating Hormone
MMPs	Matrix Metallopeptidases
LOX	Lysyl Oxidase
ATRA	All-trans Retinoic Acid
CAT	Catalase
H_2O_2	Hydrogen Peroxide
DMSO	Dimethyl Sulfoxide
TdT	Terminal Deoxynucleotidyl Transferase
BCL2	B Cell Lymphoma2
COX-2	Cyclooxygenase 2

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